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Cancer 73 (3 Suppl) pages 1114-20 Feb 1 1994

Cancer Research 53(17) pages 3956-63 Sep 1, 1993

Bioconjugate Chemistry 3 (1) pages 42-8 Jan-Feb 1992

Cancer Immunology, Immunotherapy 34 (5) pages 343-8 1992

Thanks.

Jennifer Hunt

Patent Examiner, Art Unit 1642

CM1-8D06

(703)308-7548

## Site-specific Prodrug Activation by Antibody- $\beta$ -Lactamase Conjugates: Regression and Long-Term Growth Inhibition of Human Colon Carcinoma Xenograft Models

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Hybritech Incorporated, San Diego, California 92196-9006 [D. L. M., S. D. M., D. G. M.], and Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285 [L. N. J., K. L. L., T. A. S., S. L. B., J. J. S.]

### ABSTRACT

Antibody-directed catalysis (ADC) is a two-step method for the delivery of chemotherapeutic agents in which enzyme-antibody conjugate, prelocalized to antigen-bearing tumor cells, catalyzes the site-specific conversion of prodrug to drug. An ADC system consisting of F(ab')<sub>2</sub>- $\beta$ -lactamase conjugates and a cephalosporin derivative of the oncolytic agent 4-desacetylvinblastine-3-carboxyhydrazide was investigated.

The ability of the system to mediate antitumor activity was compared with that of free drug given alone and with covalent drug-antibody conjugates in LS174T and T380 colon carcinoma xenografts in nude mice. Efficacy increased from moderate tumor growth inhibition by using free 4-desacetylvinblastine-3-carboxyhydrazide to tumor regression and long-term stabilization with the ADC system. Labile covalent drug-antibody conjugates prepared from the same antibodies were less effective than ADC and required much higher antibody doses.

The antigens KSI/4, carcinoembryonic antigen, and tumor-associated glycoprotein-72, TAG-72, present on the model cell lines, were chosen to investigate the effect of differences in subcellular location and expression heterogeneity on the efficacy of ADC delivery. Response was equivalent with the three tumor antigens. Hence, heterogeneous expression and membrane shedding of carcinoembryonic antigen and TAG-72, did not diminish the suitability of these antigens as targets for ADC therapy. In contrast, drug-antibody conjugate efficacy was more sensitive to subcellular location and heterogeneity.

Thus, ADC is a highly effective form of immunochemotherapy in preclinical models, with applicability toward a variety of antigen targets.

### INTRODUCTION

The fundamental rationale for targeted delivery systems is that efficacy can be maximized and toxicity minimized by biasing the biodistribution of the chemotherapeutic agent toward the disease site and away from sites of organ toxicity. To achieve advantage over more traditional forms of treatment, a targeted delivery system must also produce a higher concentration of drug at the tumor than can be produced by nontargeted administration of the same drug. Several recent reports (1-3) have described ADC<sup>2</sup> systems which attempt to provide, simultaneously, a biodistribution bias favoring the tumor, and a mechanism for obtaining higher tumor concentrations of drug than could be obtained by direct administration of active agent. This is achieved by using an enzyme covalently conjugated to a tumor-selective antibody. Such a conjugate catalytically converts an independently administered prodrug into the active oncolytic agent, potentially reaching a concentration far greater than that of the conjugate itself.

The current study was undertaken to determine whether ADC is capable of overcoming two of the most important theoretical limitations of targeted delivery with drug-antibody covalent conjugates: the

requirement for large antibody doses and the paucity of antigens that are both sufficiently specific for effective targeting and sufficiently concentrated at the tumor to mediate delivery of therapeutic concentrations of drug (4). To this end experiments were performed to determine: (a) the pharmacokinetic behavior of an enzyme-antibody conjugate; (b) the efficacy of the ADC system directed toward three different antigens in two different cell lines, relative to nontargeted drug treatment, and to covalent drug-antibody conjugate treatment with the same drug; and (c) the minimum conjugate dose required to obtain maximal efficacy.

The primary goals in selecting the enzyme and substrate components of this ADC system were (a) that the enzyme and substrate be absent from mammalian tissues; (b) that the system offer potential for release of a variety of drugs with drug release mediated via several different functional groups; (c) that the drug released (at least in initial studies) not require further activation or metabolism; and (d) that the molecular weight of the conjugate be minimized in order to facilitate rapid clearance of the conjugate from circulation. The enzyme chosen to meet these criteria was the  $\beta$ -lactamase from the 265A strain of *Enterobacter cloacae*, and the prodrug was a cephalosporin sulfoxide derivative of 4-desacetylvinblastine-3-carboxyhydrazide. Similar considerations have also led others to adopt P99 or other  $\beta$ -lactamases for site-specific prodrug activation (5-7).

The *E. cloacae* 265A  $\beta$ -lactamase, designated P99, was selected because of its ability to hydrolyze a large number of cephalosporins. The P99 enzyme has been well studied (8, 9). Its amino acid and gene sequences have been determined and the active site residues are known. Several of its properties were considered to be advantageous for ADC: it is of moderate molecular weight (40,000); is readily available via one-step affinity chromatography (10), and requires no cofactors. The enzyme catalyzes the hydrolysis of cephalosporins resulting in expulsion of substituents, if they are sufficiently good leaving groups (11, 12), from the cephalosporin 3' position.

To facilitate rapid clearance from the circulation, conjugates were designed with a single F(ab')<sub>2</sub>-binding arm. Use of this fragment for the antibody moiety allowed regiospecific conjugation through the hinge region sulfhydryls and facilitated purification by gel permeation chromatography (13). Hence, the studies reported here also address the question of whether single binding arm conjugates are capable of mediating this form of targeted delivery.

### MATERIALS AND METHODS

**Prodrug Synthesis.** Synthetic methodology was developed to attach the acyl hydrazide functionality of the potent vinblastine derivative DAVLBHYD to the cephalosporin 3' position via an azacarbamate linkage (Fig. 1) (14). The synthesis began with the commercially available cephalosporin, cephalothin, which was converted to the 3-(hydroxymethyl)-2-cephem derivative in two steps (15). The carboxylic acid function was protected as a benzhydryl ester, and the 3' hydroxyl moiety was acylated with *p*-nitrophenyl chloroformate to form the carbonate. Oxidation of the sulfide, with concomitant olefin isomerization, provided the 1- $\beta$ -sulfoxide-3-cephem.

Received 1/12/93; accepted 6/18/93.

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<sup>2</sup> The abbreviations used are: ADC, antibody-directed catalysis; DAVLBHYD, 4-desacetylvinblastine-3-carboxyhydrazide; CEA, carcinoembryonic antigen; MoAb, monoclonal antibody; hCG, human chorionic gonadotropin.

<sup>3</sup> T. Parr, private communication.

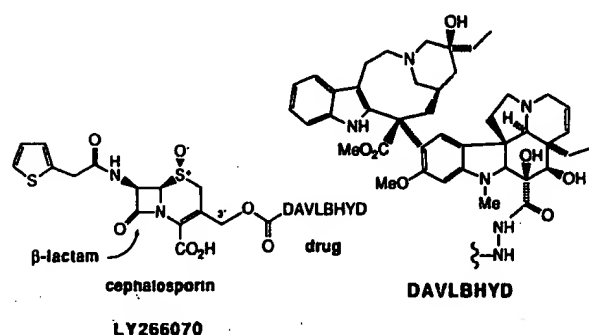


Fig. 1. Structure of the prodrug LY266070 and the oncolytic agent DAVLBHYD. Hydrolysis of the  $\beta$ -lactam ring of the cephalosporin sulfoxide results in expulsion of the 3' substituent leading to generation of DAVLBHYD.

Selective nucleophilic displacement of the carbonate with DAVLBHYD (16, 17) formed the azacarbamate linkage, thus coupling the oncolytic agent to the cephalosporin. Acid-catalyzed hydrolysis of the ester provided the desired prodrug. Previous experimentation (14) had shown that the sulfoxide form of the cephalosporin would be a better substrate with hydrolytic stability at least comparable to the unoxidized cephalosporin. The azacarbamate functional group is relatively resistant to uncatalyzed hydrolysis at physiological pH, but behaves as a leaving group after enzyme catalyzed hydrolysis of the  $\beta$ -lactam.

The molecular weight ratio, DAVLBHYD/LY266070 is 0.6. Hence, 4.0 mg/kg prodrug is equivalent to 2.4 mg/kg drug.

**Construction of F(ab') $\beta$ -Lactamase Conjugates.** Antibody-enzyme conjugates were constructed from F(ab') fragments ( $M_r \sim 50,000$ ) for targeting. Fragments were obtained by pepsin digestion of antibodies directed to three human tumor-associated antigens: KS1/4, CEA, and TAG-72 [antibodies: (K07B) (18), CEM231 (19), and CC-49 (20), respectively]. While each of these antigens is expressed on certain carcinomas, their distribution within tumors differ (see "Results"). An additional conjugate was prepared from an irrelevant antibody F(ab') fragment that recognizes no antigen in the mouse or tumor line, to show the effect of "nonspecific" conjugate. The conjugation methodology has been described (13). P99  $\beta$ -lactamase was treated with sulfo-succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (Pierce) to produce a specified level of maleimide derivatization. The maleimido groups reacted directly with the hinge region sulfhydryls of the F(ab'). This conjugation methodology preserves both antibody immunoreactivity and enzyme activity (13).

**Construction of Drug-Antibody Conjugates.** Preparation of drug-antibody conjugates has been described previously (21). Briefly, intact antibodies were oxidized with sodium metaperiodate to generate aldehyde functional groups; then purified by size exclusion chromatography. Incubation of the oxidized antibody with DAVLBHYD generated hydrazone linkages between drug and antibody. Conjugates were purified by gel chromatography, and immunoreactivity was confirmed by an enzyme-linked immunosorbent assay-based competition assay with unconjugated antibody on whole LS174T cells. Drug/antibody conjugation ratios, determined spectrophotometrically, were:  $\alpha$ -KS1/4, 5.3;  $\alpha$ -TAG-72, 10;  $\alpha$ -CEA, 2.5; IgG, 4.8.

**Labeling and Biodistribution Studies.** The biodistribution of the anti-KS1/4- $\beta$ -lactamase conjugate was determined by following  $^{125}$ I-labeled conjugate in tumor-bearing nude mice. The conjugate was first reacted with isothiocyanatobenzyl diethylenetriaminepentaacetic acid, then labeled according to established procedures (22). The labeled conjugate (20  $\mu$ g and 10  $\mu$ Ci/animal) was injected into the tail vein of nude mice with LS174T tumors. The animals were separated into groups of six, and groups were sacrificed at 4, 24, 48, 72, and 120 h after conjugate injection. Procedures for sacrificing, specimen collection, counting, and data reduction have been described previously (23). Tumors were implanted by s.c. inoculation of  $1 \times 10^7$  cells in the flank of female athymic nude mice (Charles River Breeding Laboratories, Wilmington, MA). LS174T tumors reached a mean size of 0.2 g in 9 days.

**Tumor Therapy Protocol Design.** The initial tumor therapy experiments were designed to show whether ADC delivery could immunospecifically enhance the efficacy of DAVLBHYD in two cell lines. Subsequent experiments were designed to determine which antigen best mediated the enhanced effect, what dose of antibody-enzyme conjugate was required to obtain the maximum

effect, and how ADC compared with labile covalent drug-antibody conjugate-targeted delivery in terms of overall efficacy, dependence on a particular antigen, and antibody dose requirement. Comparison of ADC delivery with covalent drug-antibody conjugate delivery was performed on the basis of an ADC-dosing protocol and a drug-antibody conjugate-dosing protocol.

**ADC Tumor Therapy Studies.** The cell lines used for *in vivo* studies are colon carcinoma lines designated T380 and LS174T. LS174T cells have been shown to express the KS1/4 (24), TAG-72 (25, 26), and CEA (27-29) antigens. CEA has also been detected on T380 cells (30). Antigen expression on the LS174T cells is described in "Results."

Tumor growth experiments were performed somewhat differently for the two tumor types. LS174T tumors (Figs. 3 and 6) were implanted as in the biodistribution study (s.c. inoculation of  $1 \times 10^7$  cells) and allowed to reach the desired size before dosing was initiated. Animals (10 in the saline control group; 5 in the treatment groups) were treated with specific ( $\alpha$ -KS1/4-,  $\alpha$ -CEA-, or  $\alpha$ -TAG-72- $\beta$ -lactamase) or nonspecific conjugate (35  $\mu$ g), or phosphate-buffered saline (10 mM NaPO $_4$ , 150 mM NaCl, pH 7.4) on the days indicated in the figures. These injections were followed after 96 h by a single injection of prodrug (4 mg/kg), drug (2.4 mg/kg), or phosphate-buffered saline. Tumor masses were calculated from tumor volume (assuming a tissue density of 1 g/cm $^3$ ), which was determined by caliper measurements, using the formula  $\text{mass} = 1 \text{ g/cm}^3 \times 0.5 \times L \times W^2$ , where  $L$  and  $W$  are the longest dimension and its perpendicular in cms, respectively. All injections were made in the tail vein.

T380 tumors (Figs. 4 and 5) were implanted on day 0 by s.c. inoculation of a 0.1 ml tumor slurry, prepared by suspending 1 g of fresh tumor from a "passage" mouse in 1 ml buffer, in the flank of female athymic nude mice. Tumors reached the desired size in 2 weeks. Treatment groups of 8 mice each were given injections of specific ( $\alpha$ -CEA- $\beta$ -lactamase) or nonspecific conjugate (35  $\mu$ g, except in the experiment of Fig. 5 wherein conjugate was administered at 3.5, 14, or 35  $\mu$ g/course of therapy, or 35  $\mu$ g in the first course of therapy only; see Fig. 5 legend), or saline (50 mM sodium borate, 100 mM NaCl, pH 8.0) on days 14, 21, and 28. These injections were followed by four daily injections of 1 mg/kg/day of prodrug LY266070 beginning 72 h after conjugate injection. In control arms, prodrug injections were substituted with either saline (150 mM NaCl) or DAVLBHYD at the molar equivalent of the prodrug dose (0.6 mg/kg/day).

All conjugate and prodrug injections were given i.v. All animals tolerated the multiple injections in fractionated dosing protocols without apparent toxicity.

**Covalent Conjugate Tumor Therapy Studies.** Drug-antibody conjugates were studied in nude mice bearing LS174T tumors in the "ADC protocol" (Fig. 7) and in a "covalent conjugate protocol" (Fig. 8). Under the ADC protocol mice were dosed starting on day 16 after tumor implantation, 96 h after saline injections. Drug-antibody conjugate doses were sized to keep the level of DAVLBHYD constant at 2.4 mg/kg/injection between experiment arms. Under the covalent conjugate protocol nude mice bearing established LS174T tumors were given injections twice per week for 2 weeks with sufficient conjugate to make the DAVLBHYD dose 2 mg/kg/injection, or with DAVLBHYD or saline. Doses of drug-antibody conjugate (in mg) administered to provide the desired level of drug are shown in Table 1.

**Data Analysis.** Rate of tumor growth was the indicator of activity used to evaluate the ADC delivery system. Efficacy was inferred from either regression of established tumors or decrease in tumor growth rate. Treatment arms were considered to be significantly different only when the standard error bars did not overlap. Statistical significance of tumor regression was indicated by nonoverlapping standard errors between earlier and later time points in a single group of mice.

## RESULTS

The two cell lines used in this study, LS174T and T380, have served as models for localization and treatment with various antibody and

Table 1. Doses of drug-antibody conjugate (in mg) administered to provide desired level of drug

	$\alpha$ -KS1/4	$\alpha$ -CEA	$\alpha$ -TAG-72	IgG
Fig. 7	1.9	4.3	1.0	2.2
Fig. 8	1.6	3.6	0.8	1.8

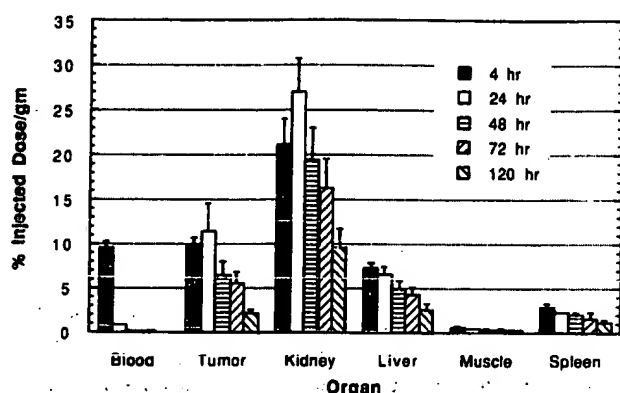


Fig. 2. Pharmacokinetics of the  $\alpha$ -KS1/4- $\beta$ -lactamase conjugate in nude mice bearing LS174T tumors.

conjugate constructs in the past. Previous studies have shown that neither KS1/4 (31) nor TAG-72 internalize efficiently (25, 26). Other studies have shown that TAG-72 and CEA antigen expression can be markedly affected by whether the cells are grown *in vitro* or *in vivo* as human tumor xenografts (32). We therefore determined the relative staining intensity and heterogeneity of all three antigen systems in LS174T tumor xenografts. Frozen sections of s.c. implanted LS174T xenografts were subjected to standard immunoperoxidase staining techniques by using biotinylated MoAbs directed against the antigen epitopes utilized in the therapy experiments described throughout this paper. An irrelevant biotinylated MoAb was also included as a negative control. This study demonstrated that the antigen expression detected by the MoAbs used to construct the  $\beta$ -lactamase conjugates was similar to that in previously published reports. Thus, the KS1/4 antigen was characterized by homogeneous, intense staining, which was similar to that observed against human colon tumor tissue (24). CEA antigen expression was observed to be intense cytoplasmic and membrane staining in 70–80% of the tumor cells, in good agreement with the findings of Esteban *et al.* (29) who examined the reactivity of the ZCE025 anti-CEA MoAb in LS174T tumor xenografts. ZCE025 binds to the same epitope as the CEM-231 MoAb used in the present study (33). Similar staining was observed with the anti-CEA antibody CEM231 on T380 tumors.<sup>4</sup> The anti-TAG-72 MoAb, CC-49 (20, 34), bound to approximately 50–70% of the LS174T tumor section and demonstrated reactivity which was associated with tumor cells as well as extracellular mucin. The binding characteristics of CC-49 against LS174T xenografts was very similar to that reported for another anti-TAG-72 MoAb, B72.3, against this same tumor (25).

Before testing the effects of ADC treatment on tumor growth it was necessary to show that tumor localization of the antibody-enzyme conjugate occurred, and to determine an appropriate prelocalization time. Tissue distribution of radiolabeled,  $\alpha$ -KS1/4- $\beta$ -lactamase conjugate (Fig. 2) showed substantial localization to LS174T tumors, with favorable ( $\geq 10$ ), tumor:blood ratios beyond the 24-h point. Within 72 h radiolabeled conjugate was barely detectable (0.1% of the injected dose) in the blood. Consequently, 72 or 96 h was considered to be a sufficient interval between conjugate and prodrug injections. The apparent decrease in tumor conjugate concentration between 72 and 120 h (Fig. 2) was shown to be due to increase in tumor mass during this period, not conjugate egress (see "Discussion"). Conjugate concentration in serum has also been followed by enzyme assay (35) and was shown to drop to an acceptably low level (200 ng/ml) within 72 h after injection.

*In vivo* antitumor activity of the various ADC treatment regimens is shown in Figs. 3–6. Antibody-enzyme conjugate administration is indicated by open arrows and prodrug administration by filled arrows. Standard errors are included with all measurements to indicate statistical significance. Dose schedules were not optimized; instead, controls were included in each experiment to ensure that proper conclusions could be drawn.

Antibody-enzyme conjugate doses were based on a preliminary dose-ranging experiment (not shown) with 35- and 280- $\mu$ g doses of conjugate and 4 mg/kg LY266070. Equivalent weight loss and tumor response at the two doses suggested that all prodrug was converted to drug with the 35- $\mu$ g conjugate dose.

The prodrug dose and schedule used for the ADC experiments shown in Figs. 3 and 6, i.e., 4-mg/kg bolus i.v. injection once weekly for 3 consecutive weeks, were chosen because this was shown in preliminary experiments (not shown) to be the maximum amount of prodrug that could be administered by itself which did not result in significant antitumor efficacy or animal toxicity. The equivalent amount of free DAVLBHYD (2.4 mg/kg), used in Figs. 3 and 7, was also near its maximum tolerated dose since free drug treatment caused the mice to lose between 13 and 15% of their pretreatment body weight. In contrast, the weight loss due to the 4-mg/kg prodrug treatment ranged from 7 to 10%. The prodrug dose was fractionated in the experiments of Figs. 4 and 5 to determine whether fractionation could be used to decrease treatment toxicity, thereby permitting higher prodrug doses. The results supported the hypothesis, in that neither the mice treated with free drug nor the mice treated with conjugate/prodrug exhibited any weight loss under this protocol, while efficacy appeared to have been similar to that seen with bolus dosing (see below).

ADC treatment of LS174T tumors with either CEA- or KS1/4-targeted conjugates and prodrug caused the tumors to regress. Using  $\alpha$ -KS1/4 conjugate, tumors regressed from  $0.37 \pm 0.17$  (SE) g on day 16 to  $0.01 \pm 0.01$  g on day 44, while with  $\alpha$ -CEA conjugate the tumors regressed from  $0.30 \pm 0.15$  to  $0.05 \pm 0.03$  g (Fig. 3) over the same period. These growth curves show that while antigen-specific ADC treatment caused regression of tumors, treatment with free drug at the same dose caused only a delay of about 23 days in the time required for the tumors to reach 2 g. Growth curves for CEA- and KS1/4-directed treatments were indistinguishable. Prodrug alone had little effect on tumor growth. Irrelevant conjugate/prodrug had activity at least as great as free drug alone, but significantly less than antigen-targeted treatments, indicating that antigen specificity contributes to treatment efficacy.

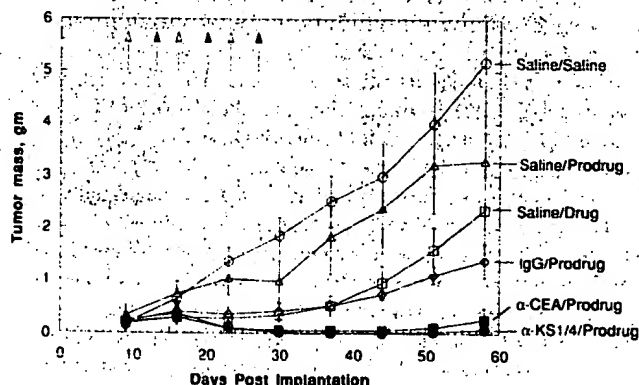


Fig. 3. LS174T tumor growth curves following ADC treatment. Administration of conjugate, 35  $\mu$ g of  $\alpha$ -KS1/4,  $\blacksquare$ , or  $\alpha$ -CEA,  $\blacksquare$ , or irrelevant,  $\diamond$ , open arrows, was followed after 96 h by 4.0 mg/kg of LY266070, filled arrows. Additional control groups were treated with saline followed by the same dose of LY266070,  $\Delta$ , by the molar equivalent dose of DAVLBHYD, 2.4 mg/kg,  $\square$ , or by saline,  $\circ$ , on the same schedule. Tumor masses averaged 0.2 g at initiation of treatment.

<sup>4</sup> D. Mackensen, unpublished results.

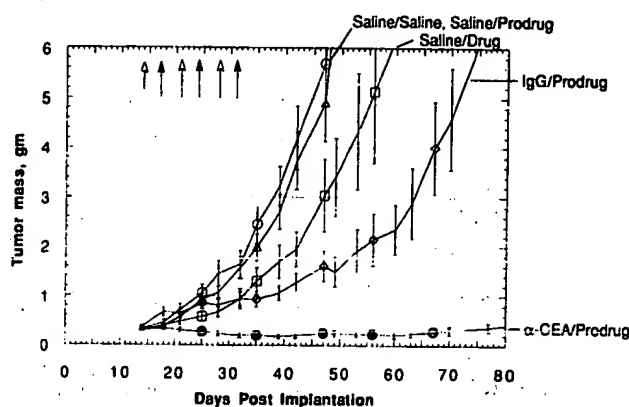


Fig. 4. T380 tumor growth curves following ADC treatment directed at CEA. Treatments were similar to those in Fig. 3, except that prodrug administration was begun 72 h after conjugate instead of 96, and was fractionated over 4 days. Thus, conjugate administration, 35  $\mu$ g of  $\alpha$ -CEA,  $\bullet$ , or irrelevant,  $\circ$ , open arrows, was followed after 72 h by 1.0 mg/kg/day of LY266070 for 4 days. The filled arrows denote the first day of prodrug dosing. Additional control groups were treated with saline followed by LY266070,  $\Delta$ , DAVLBHYD,  $\square$ , or by saline,  $\circ$ , on the same schedule. Tumor masses averaged 0.3 g at initiation of treatment.

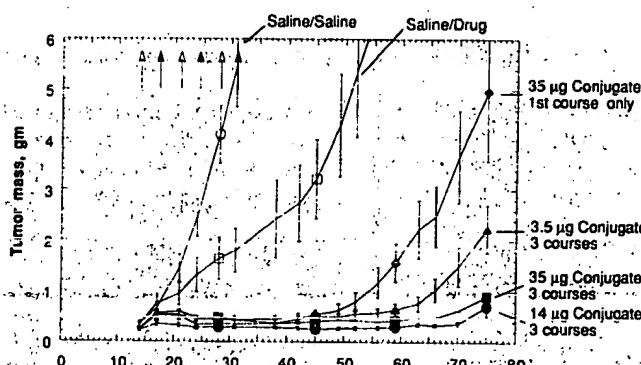


Fig. 5. Response of T380 tumors to ADC treatment with different doses of  $\alpha$ -CEA- $\beta$ -lactamase conjugate. Treatment schedule was identical to that in Fig. 4, e.g., conjugate followed after 72 h by 4.0 mg/kg prodrug fractionated over 4 days. Conjugate was administered at 35,  $\blacksquare$ , 14,  $\bullet$ , and 3.5,  $\blacktriangle$ ,  $\mu$ g in each course of therapy. A fourth group of mice,  $\diamond$ , were treated with 35  $\mu$ g conjugate in the first therapeutic course only, e.g., on day 14 only, but with prodrug in all three courses. Control groups were treated with saline, followed by DAVLBHYD,  $\square$ , or saline,  $\circ$ , on the same schedule. Tumor masses averaged 0.3 g at initiation of treatment.

T380 xenografts also regressed, from  $0.34 \pm 0.03$  g on day 18 to  $0.22 \pm 0.07$  g on day 60, on treatment with  $\alpha$ -CEA- $\beta$ -lactamase conjugate and LY266070 (Fig. 4; note that the solid arrows in the figure denote the first 4 days of prodrug dosing in each course of therapy as described in "Materials and Methods"). As in the LS174T model free DAVLBHYD produced only a minor growth delay (9 days in the time required for tumors to reach 2 g), while ADC treatment produced regression and long-term stabilization of tumor mass with an equivalent drug dose. Irrelevant conjugate/LY266070 had an intermediate effect in which growth to reach 2 g was delayed 26 days without regression.

The relationship between antibody-enzyme conjugate dose and response was examined by holding the prodrug dose constant at 4 mg/kg/course of therapy fractionated over 4 days as in the experiment of Fig. 4, and decreasing the  $\alpha$ -CEA- $\beta$ -lactamase conjugate dose. An additional treatment group received conjugate in the first treatment course only, but received prodrug in all three courses, and a control group received free drug at a molar equivalent dose. Growth curves are compared in Fig. 5. All ADC treatment groups showed initial tumor regression, but the duration of the response varied with conjugate dose. Responses duplicating those observed in Fig. 4 were ob-

served in response to ADC treatment with 35 and 14  $\mu$ g conjugate/course, while the duration of response to 3.5  $\mu$ g/course was shorter. Thus, maximal efficacy was obtained at between 3.5 and 14  $\mu$ g  $\alpha$ -CEA- $\beta$ -lactamase conjugate/course.

Administration of conjugate with only the first course caused a diminished effect in which tumor growth was delayed 39 days, compared to saline-treated controls, in the time required to reach 2 g. Histological examination of the small tumor areas present at various stages of treatment in additional sets of mice showed that CEA was available to bind the second and third courses of conjugate (data not shown).

Coexpression of KS1/4 (24, 36), CEA (30, 37), and TAG-72 (25) antigens (see above) by LS174T tumor cells enabled the direct comparison of two modes of targeted chemotherapy on a single cell line: ADC (Fig. 6) and covalent drug-antibody conjugates (Figs. 7 and 8). For the ADC study doses and schedule were identical to those in the experiment of Fig. 3. Large initial tumor volumes averaging 0.6 to 0.7 g were used in these experiments to provide a significant challenge to treatment. Tumors in animals treated with  $\alpha$ -CEA- $\beta$ -lactamase/prodrug regressed from  $0.83 \pm 0.24$  g on day 20 to  $0.19 \pm 0.07$  g on day 41;  $\alpha$ -KS1/4- $\beta$ -lactamase/prodrug-treated tumors changed from  $0.96 \pm 0.23$  g to  $0.49 \pm 0.26$  g over the same period; while tumors treated with  $\alpha$ -TAG-72- $\beta$ -lactamase/prodrug changed from a maxi-

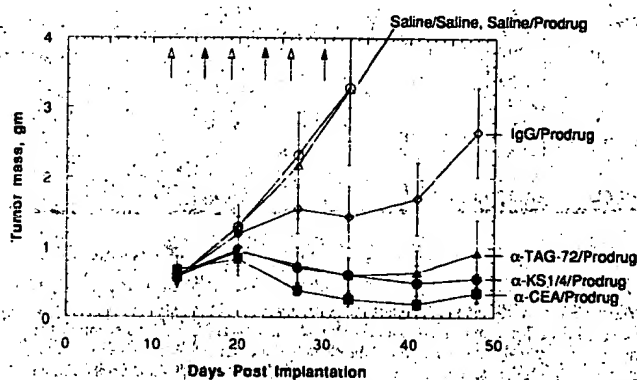


Fig. 6. Response of LS174T tumors to ADC treatment directed to three different antigens.  $\beta$ -Lactamase conjugates of  $\alpha$ -CEA,  $\blacksquare$ ,  $\alpha$ -KS1/4,  $\bullet$ ,  $\alpha$ -TAG-72,  $\blacktriangle$ , or irrelevant,  $\circ$ , antibodies, administered at a dose of 35  $\mu$ g, open arrows, were followed after 96 h by 4 mg/kg LY266070, filled arrows. Additional control groups were treated with saline followed by LY266070,  $\Delta$ , or saline,  $\circ$ , on the same schedule. Tumor masses averaged 0.6 g at initiation of treatment.

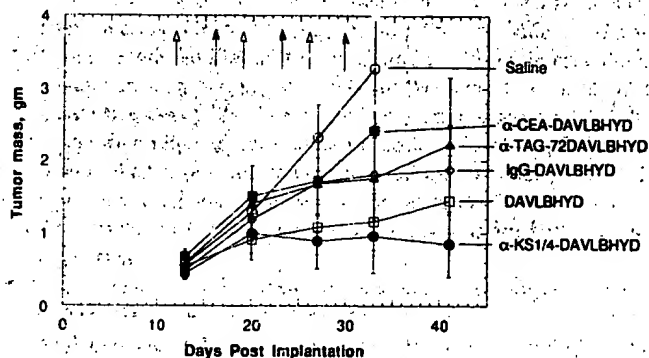


Fig. 7. Response of LS174T tumors to drug-antibody conjugate treatment directed to three different antigens. DAVLBHYD conjugates of  $\alpha$ -CEA,  $\blacksquare$ ,  $\alpha$ -KS1/4,  $\bullet$ , and  $\alpha$ -TAG-72,  $\blacktriangle$ , or irrelevant antibodies,  $\circ$ , were administered to mice, filled arrows, 96 h after saline injections, open arrows, mimicking the ADC dose schedule of Fig. 6. Additional control groups were treated with saline followed by DAVLBHYD,  $\square$ , or saline,  $\circ$ , on the same schedule. Since the drug/antibody values differed for the three conjugates, different conjugate doses were administered to supply an equal amount of DAVLBHYD: 2.4 mg/kg. Conjugate doses are given in "Materials and Methods." Tumor masses averaged 0.6 g at initiation of treatment.

num of  $0.94 \pm 0.27$  g on day 20 to  $0.60 \pm 0.26$  g on day 33. Growth curves are compared in Fig. 6. While no significant difference between the targeted treatment groups was observed, only the  $\alpha$ -CEA- $\beta$ -lactamase/prodrug treatment strictly met the criterion for regression, as described in "Materials and Methods." Irrelevant antibody- $\beta$ -lactamase conjugate/prodrug treatment resulted in an intermediate effect in which growth was delayed (18 days to reach 2 g) but no regression was observed.

Treatment with DAVLBHYD delivered as a covalent drug-antibody conjugate was measured for comparison with ADC delivery by using  $\alpha$ -KS1/4-,  $\alpha$ -CEA-, and  $\alpha$ -TAG-72-DAVLBHYD conjugates. Doses were calculated to provide the same molar quantity of DAVLBHYD as the ADC treatments (see "Materials and Methods"). The growth curves (Fig. 7) show that no tumor regression occurred in any treatment arm and that only KS1/4-DAVLBHYD may have been more effective than irrelevant conjugate, although the difference did not meet the criterion for significance.

An additional experiment was performed to test the efficacy of the covalent drug-antibody conjugates in a more intense dose schedule, typical of previous experiments with these agents (25). Mice with initial tumor burden averaging 0.5 g were given injections twice per week for 2 weeks of sufficient  $\alpha$ -KS1/4 and  $\alpha$ -TAG-72 drug-antibody conjugate to give 2 mg/kg DAVLBHYD per injection. Mice with initial tumor burdens of 0.15 g were treated with  $\alpha$ -CEA-DAVLBHYD conjugate or the identical control materials. In one set of animals treated with free drug the dose was 60% lethal dose, in the other it was 40% lethal dose. Growth curves (Fig. 8) show no tumor regressions. KS1/4- and TAG-72-DAVLBHYD conjugates appeared to suppress LS174T tumor growth relative to the saline-treated control group (Fig. 8A). Neither of these conjugates, however, was signifi-

cantly more effective than an irrelevant IgG-DAVLBHYD conjugate (Fig. 8A). The anti-CEA-DAVLBHYD conjugate, on the other hand, was more effective than either free DAVLBHYD or an irrelevant IgG-DAVLBHYD conjugate when used in a lower tumor burden model (Fig. 8B).

## DISCUSSION

**Tumor Growth Inhibition.** The data in Figs. 3–6 demonstrate that the activity of the oncolytic agent DAVLBHYD can be qualitatively increased from moderate tumor growth inhibition to tumor regression and long-term stabilization by utilizing the antibody-directed catalysis delivery system. Since this enhancement of activity was observed in both the T380 and LS174T colon carcinoma models it is unlikely that the result is strongly cell line dependent.

The data in Figs. 3 and 6 show that the ADC delivery system mediates tumor regression with the three antigen targets, CEA, KS1/4, and TAG-72, which have different patterns of expression. The choice of antigen to be utilized for antibody-targeted therapy is generally critical to its success. Many targeted delivery systems require cellular internalization for anti-tumor activity (38, 39), but only a few of the known tumor-selective antigens internalize efficiently (40). For those antigens that do internalize, each cell that the antibody fails to target, whether because of antigen heterogeneity, slow antibody diffusion rate, or poor vascularization, represents a locus from which the tumor can regenerate without inhibition.

Internalization is not required for chemoimmunoconjugates constructed with certain suitable linkers, like those used in the experiments of Figs. 7 and 8. These "labile-linked" conjugates have demonstrated preclinical activity against a variety of target antigens (31). But even for drug-antibody conjugates that do not require internalization, antitumor efficacy has been shown to correlate with target antigen expression level (41). The results in Figs. 7 and 8 showing that KS1/4 targeting is more effective than CEA or TAG-72 targeting for DAVLBHYD-antibody conjugates are in agreement with this conclusion. It would be clearly advantageous, therefore, to use a site-specific delivery system which is relatively unaffected by antigen characteristics such as copy number and heterogeneous expression, and which does not require internalization.

Anti-CEA and anti-TAG-72 conjugates do not internalize efficiently (25, 26, 30, 37). Both bind antigen in the interstitial fluid as well as at cell membranes and both antigens are heterogeneously distributed in LS174T tumors, TAG-72 being more heterogeneous than CEA. Conjugates targeting KS1/4, in contrast, bind exclusively at the cell membrane and may bind virtually every LS174T cell (see "Results"). These three antigens, therefore, represent a diverse panel of molecular targets with which to evaluate the therapeutic potential of the ADC system. The data in Figs. 3 and 6 indicate that ADC therapy was quite successful for all three antigens with no significant difference in activity among them, and is, therefore, relatively unaffected by the differing properties of these antigens.

Within this set of experiments two slightly different dosing schedules were also compared. While the total prodrug doses were the same in all experiments reported, the weekly 4-mg/kg prodrug dose was fractionated over 4 days instead of being given in a single bolus in the T380 experiments (Figs. 4 and 5). Fractionation should lead to greater specificity, because conjugate not bound in the tumor has more time to clear before the full prodrug dose is administered. Furthermore, it was reasoned that if the prodrug elimination was rapid, fractionation might result in a higher percentage of the total dose reaching the tumor, while a lower peak concentration of the drug might reduce its overall toxicity. The only clearly discernible difference between the fractionated and bolus dosing was that mice in the free drug control

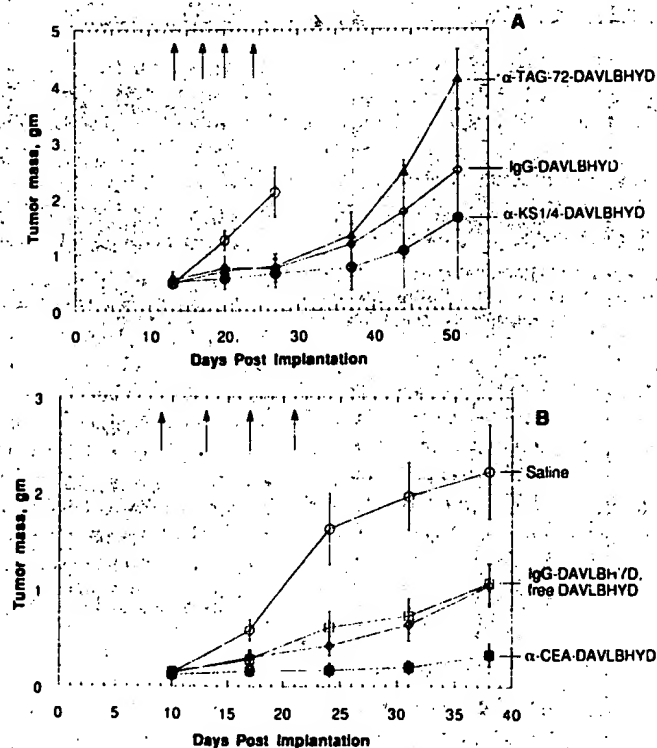


Fig. 8. Response of LS174T tumors to drug-antibody conjugate treatment on a more intense dosing schedule. DAVLBHYD conjugates of  $\alpha$ -CEA,  $\blacksquare$ ,  $\alpha$ -KS1/4,  $\square$ ,  $\alpha$ -TAG-72,  $\triangle$ , or irrelevant antibodies,  $\diamond$ , were administered twice per week for 2 weeks, filled arrows, at a dose sufficient to provide 2.0 mg/kg DAVLBHYD in each injection. Conjugate doses are described in "Materials and Methods." Additional control groups were treated with DAVLBHYD at 2.0 mg/kg/injection,  $\square$ , or saline,  $\circ$ . Initial tumor masses averaged 0.5 g in the experiment of A and 0.15 g in the experiment of B.



groups showed no weight loss with fractionated dosing but significant weight loss with bolus dosing.

The data also show that single binding arm antibody-enzyme conjugates used in the experiments of Figs. 3–6 mediate drug delivery. Since comparable results were obtained with prodrug fractionated over days 4–7 (Figs. 4 and 5) to results with prodrug given in a bolus on day 5 (Figs. 3 and 6), retention of the single binding arm conjugate in tumor appears to be adequate. However, the result obtained with injection of conjugate in the first therapeutic course only (Fig. 5, ♦) suggests that the tumor residence time of the conjugate during treatment is less than 3 weeks. Egress of conjugate from the tumor compartment during treatment could be due to the antigen-antibody-binding equilibrium, in which case a dual binding arm conjugate [e.g., an antibody- or  $F(ab')_2$ -enzyme conjugate] would be advantageous; but it could also be due in part to metabolic processes at the tumor. Physiological changes, resulting from, for instance, cell lysis, could also decrease retention of conjugate at the tumor.

It has been noted that one potential disadvantage to the use of covalent drug-antibody delivery is that high doses of antibody are likely to be required in humans (4). The doses of DAVLBHYD-antibody conjugate given in the experiments of Figs. 7 and 8 ranged from 800 to 4300  $\mu$ g. Doses of 270 to 4800  $\mu$ g/course of therapy were required to effectively treat LS174T (25, 33) and other (41) tumor xenografts with labile-linked chemoimmunconjugates in previous studies. The experiment of Fig. 5 was performed to determine the relationship of response to antibody-enzyme conjugate dose for the ADC system. The results showed that response increased with dose up to 14  $\mu$ g, but not above this level, so that in the T380 model maximum efficacy was achieved with between 3.5 and 14  $\mu$ g conjugate. Thus, the quantity of conjugate localized at the tumor following a 14- $\mu$ g conjugate dose apparently hydrolyzed all LY266070 that reached the tumor and additional catalytic activity had no additional benefit at this prodrug dose. (In a separate test of prodrug dose response, tumor growth inhibition decreased when prodrug dose was decreased at constant conjugate level. Data not shown.)

The ratio of antibody doses used to give equivalent quantities of drug in the different delivery formats (drug-antibody conjugate/ADC) are summarized in Table 2. They vary from 16 to 69 on a molar basis depending on the number of drug residues per antibody for the covalent drug-antibody conjugates. These ratios are calculated from the doses actually used in the experiments of Figs. 6 and 7, and are artificial in the sense that drug dose for ADC can be increased without increasing the antibody-enzyme conjugate dose. Since the data in Fig. 5 show that considerably less than 35  $\mu$ g of  $F(ab')_2$ - $\beta$ -lactamase may be required to achieve the full effect, it is estimated that drug-antibody conjugates require doses ranging upward from 100 times greater than required by ADC to deliver equivalent drug. Furthermore, even larger amounts of drug-antibody conjugate administered in a more intense schedule (Fig. 8) failed to produce the regressions observed with ADC treatment. Hence, these data suggest that ADC has the advantage over drug-antibody conjugate delivery that, in addition to improved efficacy, it requires much less antibody.

Table 2. Ratio of antibody doses: DAVLBHYD antibody: $\beta$ -lactamase antibody

These values are the fold excess of antibody required for delivery of equivalent quantities of DAVLBHYD (2.4 mg/kg) in Fig. 7 (drug-antibody conjugate) over treatments in Fig. 6 (ADC treatment). The fold antibody excess required for drug-antibody conjugates increases when dosing is changed to achieve better efficacy (Fig. 8), or if ADC treatment is equally effective at a lower conjugate dose, as suggested by the results in Fig. 5.

Antibody	wt/wt	mol/mol
$\alpha$ -KS1/4	53	30
$\alpha$ -CEA	123	69
$\alpha$ -TAG-72	28	16

The antibody-enzyme conjugate dose required to reach maximal efficacy in ADC is expected to be a function of the kinetic constants for the enzyme/substrate pair, and the potency of the drug. If enzyme-catalyzed hydrolysis of prodrug is slow, then a large concentration of enzyme would be required to activate sufficient drug to reach a therapeutic threshold. Likewise, at a given enzyme turnover rate, a lower potency drug should require a larger amount of enzyme to produce the therapeutic effect. The  $\beta$ -lactamase-LY266070 system of the current study involves both a relatively rapid turnover rate [ $k_{cat}/K_M \sim 11$  (s  $\mu$ M) $^{-1}$ , (13)], and a relatively potent drug (50% inhibiting concentration  $\sim 10^{-8}$  M). Hence, the conjugate dose requirements for other systems could be considerably higher (or lower) than the one determined in this study, depending on the potency of the drug and the rate at which it is produced by the enzyme. We note for comparison that antibody doses in preclinical radioimmunoimaging and radioimmunotherapy experiments performed in our laboratories typically use 10–20  $\mu$ g conjugate/mouse (42, 43). The radioimmunoimaging studies have been successfully translated into human patients by using doses between 1 and 50 mg of antibody. We predict that dose requirements would translate similarly from mice to humans for antibody-enzyme conjugates if the drug potency and enzyme kinetic parameters were similar to those of this study. If the prodrug were a significantly worse substrate (lower  $k_{cat}/K_M$ ), or significantly less potent, then higher conjugate dose requirements would be expected.

In the experiments depicted in Figs. 3 and 4, irrelevant antibody-enzyme conjugate/prodrug was more effective than a molar equivalent dose of free drug. This efficacy enhancement may be due to conjugate which localizes in tumor independent of antigen binding ["preferential accumulation" (4)]. Theoretical considerations suggest that some non-antigen-mediated localization of proteins to tumors is expected and can be attributed to tumor physiology (44).

**Pharmacokinetics.** The pharmacokinetic behavior of the  $\alpha$ -KS1/4- $\beta$ -lactamase conjugate (Fig. 2) is similar to that reported previously for  $F(ab')_2$  fragments (45–47). Rapid clearance from the blood and good tumor:blood ratios indicate favorable pharmacokinetics of the conjugate. Since the behavior of the  $\alpha$ -KS1/4 conjugate resembled closely that of other constructs of similar molecular weight, and since tumor targeting had previously been demonstrated with each of the antibodies from which the conjugates were constructed, it was assumed that the  $\alpha$ -CEA and  $\alpha$ -TAG-72 conjugates would also localize and would clear from the serum within 72 h. The data showing that the efficacy and toxicity resulting from treatment with these conjugates were no different than those resulting from treatment with  $\alpha$ -KS1/4 conjugates support this assumption.

The data reported in Fig. 2 suggest that conjugate level in the tumor decreased continuously from 24 to 120 h after injection. However, examination of the organ masses showed that the tumor mass change between 72 and 120 h accounted for essentially all of the conjugate concentration decrease during this period. Egress of conjugate from the tumor after 72 h was found to be very slow.

Reports on the pharmacokinetics of antibody-enzyme conjugates with different molecular properties have appeared previously (48). Bagshawe (3), investigating  $F(ab')_2$ -carboxypeptidase G<sub>2</sub> conjugates, noted a slow rate of serum clearance of anti-CEA conjugate relative to anti-hCG conjugate. This difference was attributed to accelerated clearance of the hCG conjugate due to complex formation between conjugate and the hCG antigen (which is secreted into the serum at high level), and subsequent removal of the immune complex by the reticuloendothelial system. Since the KS1/4 antigen is a transmembrane glycoprotein (36), it is unlikely that shedding and immune complex formation were involved with the pharmacokinetics of the  $\alpha$ -KS1/4- $F(ab')$  conjugate of Fig. 2. The relatively rapid clearance of this conjugate suggests that immune complexes are not required for



rapid serum clearance, and that other molecular properties are responsible for the slow serum clearance of the F(ab')<sub>2</sub>-carboxypeptidase G<sub>2</sub> conjugate.

**Tumor Growth Inhibition in Comparable Systems.** Antibody-directed catalysis systems based on carboxypeptidase G<sub>2</sub> (referred to by the authors as Antibody-Directed Enzyme Prodrug Therapy or ADEPT) showed efficacy against LS174T and other tumor xenografts (3). In the LS174T studies targeting the CEA antigen, tumor growth inhibition, but not regression, resulted from a three-step regimen: (a) localization of anti-CEA F(ab')<sub>2</sub>-carboxypeptidase G<sub>2</sub> conjugate; (b) galactosylated enzyme-blocking antibody; and (c) nitrogen mustard-derived prodrug. The galactosylated enzyme-blocking antibody had the effect of clearing conjugate from the serum to allow prodrug administration while tumor level of conjugate was still high (49). This study was conducted with a relatively small initial tumor volume, used a prodrug dose higher than the maximum tolerated free drug dose, and used only a single course of therapy (3). In studies targeting the hCG antigen in a different cell line, cures were reported (3).

Antibody-directed catalysis systems based on alkaline phosphatase and other enzymes have also been reported to be active in tumor xenograft models (2). Whole antibody-alkaline phosphatase conjugates were used in combination with prodrugs that are phosphorylated derivatives of etoposide and mitomycin C to obtain tumor growth inhibition. Efficacy was shown to be dramatically enhanced over treatment with free drug when prodrug doses 2- to 3-fold higher than free drug were used. However, efficacy was relatively nonspecific: 4.3 versus 3.7 log cell kill for tumor-specific versus irrelevant conjugate, respectively, in one experiment using mitomycin phosphate for prodrug. The nonspecificity was attributed to substantial conjugate remaining in the serum when the prodrug was administered, 24 h after injection (2). In all *in vivo* studies reported to date (2, 3), however, ADC delivery has proven more effective than direct injection of the free chemotherapeutic agent.

As noted, the previous studies on the efficacy of ADC delivery systems (2, 3) have used prodrug doses greater than the molar equivalent free drug dose, taking advantage of the difference in toxicity between drug and prodrug in these systems (2, 50). The remarkable enhancement for molar equivalent doses observed here with ADC delivery (Figs. 3-6) may result from an improvement in the bioavailability of the prodrug over the free drug. Pharmacokinetic studies to investigate this question are in progress. A study by Antoniow *et al.* (51) demonstrated that prodrug can have pharmacokinetic properties different from those of the parent drug. It was shown that the parent drug in that system (a nitrogen mustard) was more bioavailable than its prodrug derivative (glutamic acid amide of the nitrogen mustard).

The ultimate intent of this series of experiments is to assess the practical and theoretical advantages and disadvantages of different methods of administering toxic agents to humans with the use of preclinical models. The comparison could be based on either efficacy or toxicity, but if it is based on toxicity, diminished toxicity must lead to better treatment. Labile-linked drug-antibody conjugates in this study showed efficacy comparable to, or slightly better than direct injection of drug, but the activity was strongly antigen dependent, being most pronounced for the antigen present in highest concentration, and least pronounced for a heterogeneously expressed antigen. Drug-antibody conjugates were substantially less toxic than free drug, but efficacy comparable to or better than free drug required a very high antibody dose. ADC delivery, in contrast, showed substantial improvement in efficacy over free drug, in addition to toxicity reduction. For ADC the improvement was obtained at a much lower antibody dose, and the effect was equivalent for three dissimilar antigens. Thus, ADC improved the efficacy of the oncolytic agent and overcame

two of the most troubling aspects associated with antigen-directed delivery of chemotherapeutic agents: antibody dose and antigen suitability.

## ACKNOWLEDGMENTS

We acknowledge excellent technical contributions to this work made by Leroy Baker, Vikki Sanders, and Stephen Lowe; the anti-TAG-72 antibody was developed by Dr. Jeff Schlom. We wish to thank Drs. Neil Kay, Paula Sundstrom, and Thomas Anderson for helpful criticism of the manuscript.

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